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Microbial interactions affecting stem-end blue mold decay of 'd'Anjou' pears

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Abstract

Stem-end decay of 'd'Anjou' pears caused by *Penicillium expansum* is the common decay of fruit kept in cold storage for extended periods of time. The succulent thick stems of 'd'Anjou' pear are prone to colonization by these fungi. We isolated bacteria and yeasts from surface of pear and apple fruits and evaluated them for their ability to colonize pear stem tissue. Populations of the best bacterial and yeasts colonizers increased by more than 2 log units within 3 d at 24 °C. They were then evaluated in cold storage at 1 °C for their ability to prevent infection of pear stems by a very aggressive strain of *P. expansum*, and a weakly pathogenic strain of a *Penicillium* sp. isolated from a pear stem. Only few isolates reduced stem-end decay. Two bacteria, *Pseudomonas chlororaphis* and *Enterobacter* sp. promoted fungal infection of the stem and subsequent fruit decay. The abundance of decay promoting bacteria in some years may be responsible for the high incidence of stem-end decay in those years.
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Keywords: Stem-end decay; *Penicillium expansum*; Biological control

1. Introduction

Fruit packers consider postharvest fruit decay to be the most serious problem for long term storage of winter pears (Anonymous, 1992; Kupferman, 1995). Blue mold (caused by *Penicillium expansum*) and gray mold (caused by *Botrytis cinerea*) of 'd'Anjou' pear fruit originating from stem infections appear after several months in storage and can cause significant losses (Lennox et al., 2004). The short, fleshy stems of 'd'Anjou' pear fruit are highly susceptible to infection (Lennox and Spotts, 2004; Janisiewicz, 2005). These fungi grow slowly through the stem during the first few months of storage and invade fleshy tissue causing fruit decay. Postharvest handling practices may have a significant effect on the severity of this decay. After harvest, bins with pears may be drenched, which often is followed by packing that may last until January (Xiao and Rogers, 2004). During the drenching or packing process the fruit may be contaminated with fungal spores. Repacking of the fruit may be necessary if the incidence of decay exceeds 2%. Although fungicides have been used to control these decays, they have been only partially effective (Lennox and Spotts, 2004; Anonymous, 1992). In addition, the fungicide residue on the fruit limits the export market (Lennox et al., 2004; Gullino and Kuijpers, 1994). Biological control products (e.g. BioSave®, Jet Harvest Solutions, Orlando, FL) have been used in pear packinghouses to protect fruit against decay for the last few years. They have no residue problem because they are exempt from residue tolerance restrictions (Janisiewicz and Jeffers, 1997). BioSave® has been particularly useful against decay originating from wounded flesh tissue. It appears that its efficacy against infections originating from stems is less than from wounds (Janisiewicz, personal observation). More specialized microorganisms could provide better control of these infections. Biocontrol agents effective in colonizing stem tissue also may be effective in protecting the stem against infection by decay causing fungi.

The objective of this work was to develop a pear stem inoculation and screening procedure for selecting biocontrol agents effective against stem-end decay of pears. We used bacteria and yeasts isolated from fruit against a very aggres-

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sive isolate of *P. expansum* (MD-8) and a weakly pathogenic *Penicillium* sp. originally isolated from a pear stem.

2. Materials and methods

2.1. Pathogens

The *Penicillium expansum* isolate (MD-8) is very aggressive and was isolated from decayed apple in storage and used in previous biocontrol tests (Janisiewicz and Jeffers, 1997; Janisiewicz and Marchi, 1992). The *Penicillium* sp. isolate is weakly pathogenic and was isolated from an infected pear stem. The fungi were maintained on potato-dextrose-agar (PDA) and continued virulence was assured by periodic transfers through apple fruit. Concentrated conidial suspensions were prepared from 10-day-old cultures as previously described (Janisiewicz and Marchi, 1992). For stem inoculations in the biocontrol study, the inoculation suspensions were prepared by adding concentrated conidia suspensions to the antagonist suspensions to achieve final concentrations of 5×10^4 and 5×10^5 conidia/mL.

2.2. Aggressiveness of Penicillium isolates

'd'Anjou' pears were wound-inoculated with 25 μL of 5×10^4 or 5×10^5 conidia/mL of *P. expansum* isolate (MD-8) or the *Penicillium* sp. isolate as described previously (Janisiewicz and Roitman, 1988). The fruit were stored on fruit trays in plastic boxes for 7 d at 22 °C or for 2 months at 1 °C. After storage, decay severity was determined by measuring lesion diameters. There were three fruit per replicate and three replicates per treatment. The experiment was repeated three times under cold storage conditions.

2.3. Isolation and grouping fruit isolates

Potential antagonists were isolated from pear and apple fruit harvested from an "organic" orchard at the Appalachian Fruit Research Station in Kearneysville, WV, where trees were sprayed only with Kaolin. The fruit were collected at the time of harvest, brought to the laboratory and immediately washed with phosphate buffer on a rotary shaker and then washed again and sonicated as described previously (Janisiewicz and Roitman, 1988). The washings were plated on nutrient yeast dextrose agar (NYDA) media and plates were incubated at 24 °C for up to 7 d, as described previously (Janisiewicz and Roitman, 1988). The organisms were isolated from the plates based on visual characteristics of the colonies, purified by triple re-streaking of single colonies on the medium and separated into yeasts and bacteria based on microscopic observations as described previously (Janisiewicz and Marchi, 1992). The bacteria were divided into Gram-negative and Gram-positive strains using the KOH method (Suslow et al., 1982). Individual organisms from each group were run on appropriate BIOLOG plates (Biolog Inc., Hayward, CA). The data from each group was subjected to cluster analysis using the MicroLog 4.2 (Biolog Inc.). To reduce the number of microorganisms used in the growth and biocontrol tests, only one microorganism was selected from each cluster, except for the large clusters (with more than six isolates) where two or more isolates were selected.

2.4. Growth on pear stem ends

Isolates selected after cluster analysis were evaluated for their ability to grow on pear stem ends, the site of entry for the fungus. The abscission layer was removed with a sharp scalpel to refresh the wounds. Then the pears were placed stem up on Styrofoam fruit trays in plastic boxes. The stem ends were inoculated with 20 μL of an aqueous suspension of the tested organisms harvested from actively growing over-night shake cultures. The concentration of the microbial suspension was adjusted to 95% turbidity at 420 nm (approximately 10^7 CFU/mL for bacteria and 5×10^5 CFU/mL for yeasts). Wet paper towels were placed in the boxes and the boxes were covered with plastic wrap to maintain moisture. Populations of the organisms were recovered from the stems 1 h after inoculation (T_0) or after incubation for 3 d at 24 °C. Briefly, 1 cm of a stem end was cut and ground with a mortar and pistil in 3 mL of phosphate buffer (pH 6.8). The resulting slurry was filtered through a syringe with glass wool, diluted a hundred-fold and plated on NYDA media using an Autoplate 4000 (Spiral Biotech, Inc. Norwood, MA). The colonies were counted after 48 h incubation at 24 °C using a Qcount colony counter (Spiral Biotech, Inc.). There were six replicates per treatment.

2.5. Microbial interaction on pear stem ends

Strains that grew well on stem ends were selected for further testing to determine their potential for biocontrol of stem-end decay caused by *P. expansum* and *Penicillium* sp. The inoculation procedure for evaluating biological control potential was similar to tests for growth on stems except that the stem ends were inoculated with either *P. expansum* alone (at 5×10^4 or 5×10^5 conidia/mL) or in a mixture with the individual selected microbial strains at a concentration of 95% turbidity (T) at 420 nm. After 24 h incubation in plastic boxes at room temperature, trays with the fruit were placed in cardboard boxes and stored at 1 °C for at least 3 months before evaluating for decay incidence. The treatments were replicated three times and the experiments were conducted in triplicates. The data was combined for the statistical analysis.

2.6. Strain identification

Bacterial strains that promoted blue mold decay were kindly identified by Dr. C. Bull (USDA-ARS, Salinas, CA) using fatty acid profiles (FAMES).

3. Results

Isolations of microorganisms from apple and pear fruit resulted in a great variety of Gram-negative and Gram-positive bacteria, and yeasts. Cluster analysis of the BIOLOG data resulted in 21 clusters of Gram-negative bacteria, 14 clusters of Gram-positive bacteria, and 19 clusters of yeasts. The four strains described here were in different, average size (four to six strains per cluster) clusters.

Most of the microorganisms tested grew well on stem ends and after 3 d the populations of many increased by approximately two log units (Fig. 1). Inoculations of pear flesh tissue by *P. expansum* MD-8 and the *Penicillium* sp. resulted in developing decay on pears inoculated with either pathogen and stored for 7 d at 22 °C, but at 1 °C decay developed mainly on pears inoculated with *P. expansum* MD-8 and little on pears inoculated with the *Penicillium* sp. (Table 1).

Drop inoculation of pear stems with conidia of *P. expansum* alone followed by 24 h incubation at room temperature and storage for 3 months at 1 °C resulted in the development of pear stem-end decay on 20–40% of the fruit (Fig. 2A). However, no decay developed on pears whose stems were inoculated with conidia of the *Penicillium* sp. (Fig. 2B). Two good colonizers of stems, SP129 and SA39, when inoculated

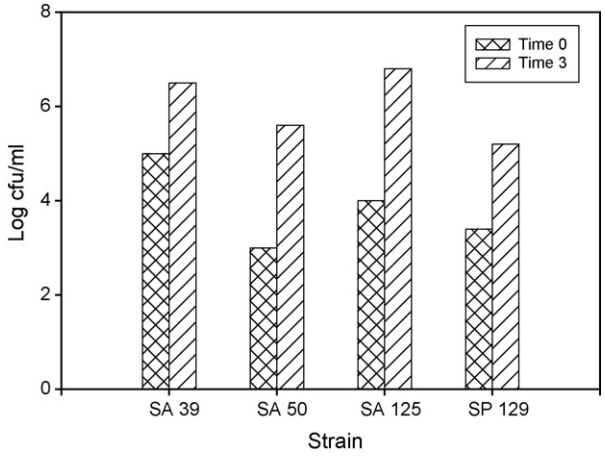

Fig. 1. Populations of potential antagonists recovered from stem ends of pears immediately after application (Time 0) and after 3 d incubation in the inoculation boxes at 24 °C.

Table 1
Lesion diameter (mm) on 'd'Anjou' pears inoculated with conidia of *Penicillium expansum* or *Penicillium* sp. and stored at 22 °C for 7 d or at 1 °C for 2 months

Pathogen	22 °C[a]		1 °C[a]	
	Pathogen concentration (conidia/mL)			
	10^4	5×10^5	10^4	5×10^5
P. expansum	34.4 a[b]	37.2 a	18.4 a	21.3 a
Penicillium sp.	11.1 b	21.4 b	0.0 b	0.9 b

[a] Storage temperature.
[b] Means within columns followed by different letter are significantly different according to *t*-test.

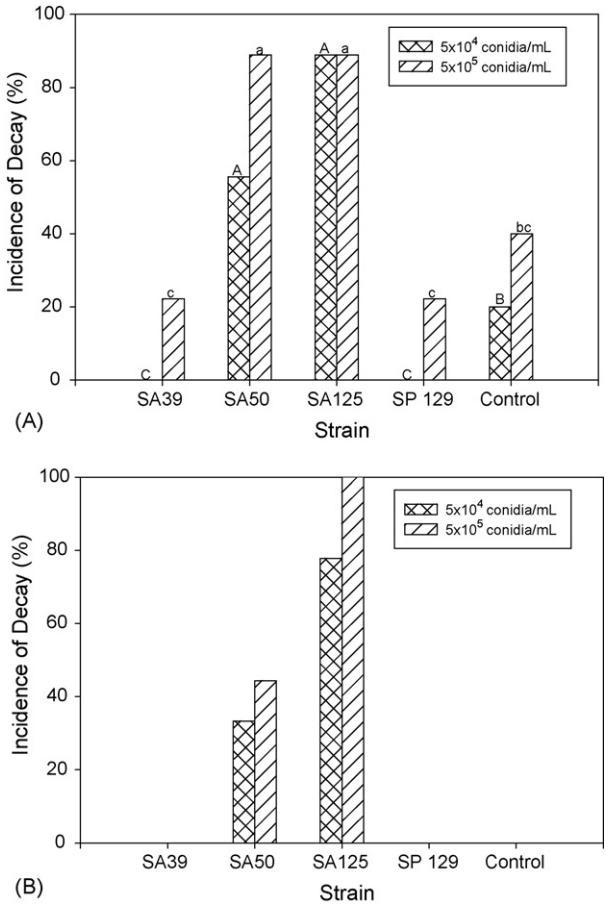

Fig. 2. Incidence of decay on 'd'Anjou' pears originating from the stem ends after 3 months of storage at 1 °C. (A) Pear stem ends inoculated with *Penicillium expansum* alone (control) or mixed with strains SA39, SA50, SA125 or SP129; (B) Pear stem ends inoculated with *Penicillium* sp. (a weak pathogen) alone (control) or mixed with strains SA39, SA50, SA125 or SP129. Note a lack of decay on fruit inoculated with conidia of the weak *Penicillium* sp. alone and moderate decay on fruit inoculated with *P. expansum* alone, and a high incidence of decay on fruit inoculated with a combinations of either pathogen and strain SA50 or SA125. Bars with different letter for each concentration of the pathogen differ according to a protected last significant difference test ($P \leq 0.05$).

onto pear stems in mixtures with a lower concentration of *P. expansum* MD-8 conidia protected fruit from blue mold decay (Fig. 2A), but other strains that colonized stems well showed little or no protective effect (data not shown). Surprisingly, inoculations of stems with a mixture of *P. expansum* MD-8 conidia (at both concentrations) and the good colonizing strains, SA50 and SA125, resulted in development of blue mold decay that was much greater than on control treatments (Figs. 2A and 3). Similarly, in an experiment with the non-aggressive *Penicillium* sp., extensive blue mold decay, up to 100% of fruit infected, developed on pears inoculated in combination with the SA125 or SA50 strains, while no decay developed on control treatments with the weak pathogen alone after 3 months in storage (Fig. 2B). These bacteria were identified by FAMES as *Pseudomonas chlororaphis* and *Enterobacter* sp. The identity of the second bacterium

Fig. 3. Incidence of decay on 'd'Anjou' pears originating from the stem ends after 3 months of storage at 1 °C. Pear stem ends were inoculated with *Penicillium expansum* (P.e.) alone (control) or mixed with strain 12 (=SA 125). P.e.1 = 5×10^5 conidia/mL, and P.e. 2 = 5×10^5 conidia/mL.

is less certain but it was tentatively identified as *Entrobacter agglomerans*.

4. Discussion

Among the natural microflora of pear and apple fruit, we found not only organisms that significantly reduced blue mold stem-end decay but also those that greatly enhanced decay. The commercial potential of the two antagonists that reduced decay warrants further investigation. This includes determination of the feasibility of large scale fermentation and formulation, then pilot testing followed by a variety of toxicological and safety tests.

The finding that two bacteria strongly promoted stem-end decay of 'd'Anjou' pears was very surprising. Incubating the fruit for an additional 5 d at room temperature further increased decay and caused a general deterioration of the entire fruit. The fact that after 3 months of storage there was no decay on fruit inoculated with conidia of the weakly pathogenic *Penicillium* sp. and up to 44–100% decay on fruit inoculated with a mixture of this pathogen and either *Entrobacter* sp. or *P. chlororaphis*, strongly suggests a stimulatory effect of these bacteria. Interestingly, both bacteria were isolated from fruit. The importance of these bacteria in promoting pear stem-end decay under commercial conditions, where there is an interaction with other microorganisms originating from fruit and soil, still must be determined.

Enhancing fruit decay by an organism that was also reported to be a good biological control agent has been reported in the past. Huang et al. (1991) demonstrated that *Burkholderia cepacia* (*Pseudomonas cepacia*) strain 2129, isolated from the surface of Washington navel oranges, enhanced green mold decay caused by *Penicillium digitatum* on orange fruit. *B. cepacia* (*P. cepacia*) is a well known biocontrol agent able to control a variety of pre- and postharvest diseases including decays on pome, stone and citrus fruits (Janisiewicz and Roitman, 1988; Smilanick and Denis-Arrue, 1992; Smilanick et al., 1993). There is a great diversity among strains of *B. cepacia* (Lessie et al., 1996; Gonzales and Vidaver, 1979), which may account for some strains producing opposite effects such as controlling or stimulating fruit decays. Similar variations may account for the stimulation of decay by *P. chlororaphis* that was reported to be an effective biocontrol agent against various diseases (Hokeberg et al., 1997; Chin-A-Woeng et al., 1998). The ability of these bacteria to stimulate decay at low inoculum concentrations (95% $T \sim 1.5 \times 10^7$ CFU/mL) and to grow well on stem ends suggests that the bacteria may contribute to the development of stem-end decay of pears under natural conditions. 'd'Anjou' pears are often drenched with suspensions containing antioxidants and decay controlling agents before placing in storage. In years with high infestations of psylla, the fruit are often covered with honeydew on which a variety of microbes thrive. During drenching, pears stem ends can be inoculated with the pathogen and other microorganisms washed from the fruit into the drenching suspension. It will be important to determine: (1) if bacteria from drenching suspensions stimulate development of stem end decay of pears, (2) if in years with a high occurrence of stem-end decay there were high counts of the bacteria in a drenching suspension, and finally

(3) what proportion of that bacteria are *P. chlororaphis* and the *Entrobacter* sp. in drenching suspensions.

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